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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ Additional inventors are being named on the separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

A NON-TOXIC SOLVENT FOR CHROMOGENIC SUBSTRATE SOLUTION AND USES THEREOF

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 16 ☐ Small Entity Statement

☒ Drawing(s) *Number of Sheets* 4 ☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☐ A check or money order is enclosed to cover the filing fee

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE [signature]

Date: November 1, 2002

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Docket Number: 13189-5USPR FC/VC

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[SOR.PAT.FORM 110 – 05/2000]

A NON-TOXIC SOLVENT FOR CHROMOGENIC SUBSTRATE SOLUTION AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

[0001] This invention relates a non-toxic solvent for preparing chromogenic substrate solution and uses thereof.

(b) Description of Prior Art

[0002] Many of the cloning and expression vectors in current use (e.g. the pUC series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the ß-galactosidase gene (lacZ). Embedded in this coding region is a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino-terminal fragment of ß-galactosidase. Vectors of this type are used in host cells that code for the carboxy-terminal portion of ß-galactosidase. Although neither the host-encoded not the plasmid-encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the lacZ gene are complemented by ß-galactosidase-negative mutants that have the operator-proximal region intact, is called α-complementation. The Lac+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-Bromo-4-chloro-3-indoxyl-ß-D-galactopyranoside (X-gal) (Horwitz et al. 1964. Substrates for cytochemical demonstration of enzyme activity. I. Some substituted 3-indoxyl-ß-D-galactopyranosides. J. Med. Chem. 7:574.). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino-terminal fragment that is not capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type. It is easily possible to screen many thousands of colonies visually and to recognize

colonies that carry putative recombinant plasmids. The structure of these plasmids is then verified by restriction analysis of mini-preparations of plasmid DNA.

[0003] To a pre-made LB agar plate containing the appropriate antibiotics, a quantity of a stock solution of X-gal (20 mg/ml in dimethylformamide (DMF) or Dimethyl sulfoxide (DMSO)) and a quantity of a solution of isopropylthio- ß-D-galactoside (IPTG) is added. The stock solution of X-gal is usually prepared by dissolving X-gal in dimethylformamide or Dimethyl sulfoxide which is a toxic solvent presenting also the drawback of providing solutions that are not stable through time.

[0004] IPTG is an important addition to the blue-white screening. The vectors carrying a segment of DNA derived from the lac operon of *E. coli* that codes for the amino-terminal fragment of ß-galactosidase can be induced by isopropylthio-ß-D-galactoside (IPTG). Bacteria exposed to the gratuitous inducer IPTG synthesize both fragments of the enzyme and form blue colonies when plated on media containing the chromogenic substrate 5-Bromo-4-chloro-3-indoxyl-ß-D-galactopyranoside (X-gal).

[0005] It would be highly desirable to be provided with new solvents that are non toxic for preparing chromogenic substrate solutions used in screening assays, these solvents providing an extended stability of the chromogenic substrate solution.

SUMMARY OF THE INVENTION

[0006] In accordance with the present invention there is provided a non-toxic dipolar solvent for chromogenic substrate for detecting presence of bacteria, which comprises a stabilizing amount of a solubilizing agent.

[0007] The solvent in accordance with a preferred embodiment of the present invention, wherein the solvent is a microemulsion.

[0008] The solvent in accordance with a preferred embodiment of the present invention, wherein the solubilizing agent is at least one selected from the group consisting of 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC) and essential oil.

[0009] The solvent in accordance with a preferred embodiment of the present invention, wherein the essential oil is present in an effective solubilizing concentration for dissolving the chromogenic substrate.

[0010] The solvent in accordance with a preferred embodiment of the present invention, wherein the essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

[0011] In accordance with the present invention, there is provided a method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce the lac operon.

[0012] The method in accordance with a preferred embodiment of the present invention, the lac operon being induced in one selected from the group consisting of *E. Coli*, Bacillus subtilis, phage, or *in situ* tissues.

[0013] In accordance with the present invention, there is provided a method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of the bacteria.

[0014] For the purpose of the present invention the following terms are defined below.

[0015] The term "chromogenic substrate" is intended to mean a substrate that produce a color when contacted with an appropriate reagent.

[0016] All references herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Fig. 1 illustrates a bacterial culture exhibiting a strong blue color indicative of lac operon induction without the presence of IPTG when X-gal dissolved in essential oils;

[0018] Fig. 2 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in dimethylformamide (DMF);

[0019] Fig. 3 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in NMP and methanol; and

[0020] Fig. 4 illustrates the results of plating a ligation/transformation onto LB plates containing X-gal dissolved in NMP, sea pine turpentine and methanol.

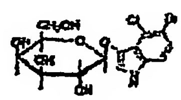
DETAILED DESCRIPTION OF THE INVENTION

[0021] In accordance with the present invention, there is provided non-toxic solvents for dissolving and stabilizing enzyme substrate used in screening assays.

[0022] One enzyme substrate widely used is X-gal, which is a dipolar molecule having the formula I:

[0023] 5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside

[0024]



I

[0025] The X-gal solutions prepared with a non-toxic dipolar solvent of the present invention, for example 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC), essential oils or a combination of these, are very stables. In solution at 4°C, the X-gal will keep its activity for more than 6 months. If used to be poured in agar plates containing the proper antibiotic, these plates will remain active and usable for at least 3 months.

[0026] As an example of its non-toxicity, NMP is known as rapidly absorbed and eliminated. It is currently used intravenously in horses as a

preanaesthetic. It is also used as an excipient in topical pharmaceutical formulations in human medicine and in cosmetics.

[0027] Essential oil mixes can be used to create a dipolar environment allowing X-gal dissolution. For instance, a combination of citrus extracts, pine terpenoids, limonene and linseed oil was shown to create an environment allowing the proper dissolution of X-gal.

[0028] Non-toxic micro emulsions can also be used to dissolve X-gal. A micro emulsion is a thermodynamically stable dispersion of one liquid phase into another, stabilized by an interfacial film of surfactant. This dispersion may be either oil-in-water or water-in-oil. Micro emulsions are typically clear solutions, as the droplet diameter is approximately 100 nanometers or less. The interfacial tension between the two phases is extremely low. Emulsions are in contrast unstable, the suspended droplets will eventually agglomerate and the dispersed phase will phase separate. Emulsion droplet sizes are much larger, typically one micron or more, resulting in a cloudy or milky dispersion. The nature of an emulsion may depend on the mixing of the ingredients and the amount of energy put into the mixing process.

[0029] A combination of essential oil was extracted with chloroform in order to remove part of the oil phase, and create a semi-precipitated emulsion. For example, 20ml of citrus extract, pine terpenoids, limonene oils and linseed oil was mixed with 20ml of Chloroform by vigorously shaking in a 50ml falcon tube. The resulting mix was let stand for approximately one day, and then the upper phase was transferred to a 15ml falcon tube. The obtained phase is a milky emulsion. It dissolves X-gal very well. If we let stand the milky phase for approximately one week, it will show the development of 5 distinct phases. Isolating each phases, it was interesting to observe that only phase # 2, the milky sub-phase, then still an emulsion, can be used as an active solution to dissolve X-gal. The active phase is yellow milky cloudy in appearance. Using 500 µl completely dissolve 0.01g of X-gal; however, it gives a very opaque and viscous solution in appearance.

[0030] Micro emulsions are proper to create dipolar environment allowing a complete dissolution of X-gal. A mixture of oil, egg yolk or

lecithine and acetic acid was sufficient to dissolve X-gal and show blue bacteria when used for its function as spread on an agar surface. Linseed oil, which has an average composition of different fatty acids (C16:0 palmitic acid 4-9%, C18:0 stearic acid 2-4%, C18:1 oleic acid 14-39%, C18:2 limoleic acid 7-19%, C18:3 limolenic acid 35-66%) is a known carrier for lipophilic molecules (as essential oils) and can act as a compound of the oily phase of the emulsion.

[0031] A micro emulsion is ideally made of a non-polar liquid mixed with a polar liquid in the presence of a surfactant or amphiphile, which is ideally a molecule carrying both polar and non-polar charges. In our situation, the surfactant, or amphiphilic molecule is X-gal. The role of the surfactant is to reduce the interfacial tension between two partially miscible or immiscible fluids below that obtained when no surfactant is present.

[0032] As well, IPTG is a usual and relatively essential addition to the cloning process. Omitting IPTG from the growth medium will decrease the expression level from plac, blue /white selection is usually not possible in the absence of IPTG. *E. coli* lac operon consists of a promoter, a transcriptional regulatory site called the operator (o), a CAP binding site (c), and three structural genes (lacZ, lacY and lacA) that are transcribed as a single polycistronic mRNA. Transcription of the lac operon is regulated by the lac repressor protein (lacI) which is encoded on a gene physically linked to the lac operon. lac operon inducers, such as IPTG, inactivate the lac repressor protein resulting in transcriptional de-repression of the lac operon. It is possible to artificially induce the lac operon using a nonmetabolizable allolactose analogue, isopropylthiogalactoside (IPTG), which binds to the lac repressor protein.

[0033] It is shown here that essential oils are replacing IPTG to induce the lac operon. It was found that compositions comprising essential oils not only dissolves X-gal, but also enhances the blue color without the need of IPTG. Among different tested essential oils, the most interesting alternative to IPTG is a small amount of the essential oil Sea Pine. Other essential oils extracted from spruce, pine or other conifers are also candidate to replace IPTG. Using such oil instead of IPTG represent

different benefits, being non-toxic, all natural, easily biodegradable, low cost and most importantly, already as a liquid solution, ready to use.

[0034] Monoterpenes and sesquiterpenes were shown to be successful in dissolving X-gal and replacing IPTG in screening clones using the blue/white X-gal selection method.

[0035] In a preferred embodiment of the present invention, individual LB plates are prepared adding 100 µl of X-gal solution to the surface of a LB plate being at room temperature and spreading evenly across the surface. The plate is dried before use. X-gal containing LB + Amp plates are stable for up to 90 days when stored at 4°C.

[0036] Batches can be prepared by aseptically adding X-gal solution directly to melted LB agar (temperature 50°C). 100 µl of the solution should be used for every Petri dish. For example, for each 500 ml add 2000 µl of X-gal solution. Mix well (for 3 to 5 minutes) and pour as you normally would. Let cool. X-gal LB plates are stable for at least 3 months when stored at 4°C. X-gal can be added before or after the addition of selective antibiotics to the medium. For best mixing results, a magnetic stir bar should be added during the autoclaving process or a sterile magnetic stir bar could be ascetically added after autoclaving.

[0037] In a preferred embodiment of the present invention, X-gal solution is prepared as follow:

<u>NMP or DMPU only</u>

[0038] Add 7 ml of either NMP or DMPU to 1 gram of X-gal. Stir until dissolved and bring to 10 ml with NMP or DMPU (whichever is already used).

<u>NMP or DMPU with essential oil</u>

[0039] Add 3.5 ml of either NMP or DMPU to 1 gram of X-gal.Stir until dissolved and bring to 5 ml with NMP or DMPU (whichever is already used). Mix with 5 ml of essential oil until thoroughly mixed.

NMP/methanol solution

[0040] Add 7 ml of either NMP or DMPU to 1 gram of X-gal and stir until dissolved. Bring to 10 ml with NMP or DMPU (whichever is already used). Add 90 ml of methanol and mix thoroughly.

Example 1

Essential oils as solvent and IPTG replacement

[0041] Using 0.01 g X-gal in 500 µl of TURPENOID NATURAL® (comprising a combination of citrus extracts, pine terpenoids, limonene and linseed oil) provides excellent dissolution of X-gal and without affecting bacterial growth. It also provides a strong blue color without the need of IPTG, as shown in Fig. 1.

Example 2

Dissolution of X-gal

[0042] 10 mg of X-gal powder were successfully dissolved in the solvents described in the table 1 below.

Table 1

Mix	T1-
Citrus lemon/Citrus sinensis	700 µl
Pinus pinaster	300 µl
Mix	**T2-**
Cupressus sempervirens	50 µl
Pinene (Sigma)	50 µl
Campher oil	100 µl
Citrus sinensis	800 µl
Mix	**T3-**
NMP	100 µl
Methanol (only to increase total volume):	*900 µl*
% NMP :	*10%*
Mix	**T4-**
PC	200 µl
Methanol (only to increase total volume):	*400 µl*
% PC :	*33%*

Example 3

Dissolution in NPM and NMP/methanol

[0043] A 10X X-gal/NMP solution could be stored at –20°C without freezing. Over time, the solution will take a very pale yellow color that does not seem to darken over time. A 10X X-gal/NMP solution is easily diluted to 1X in anhydrous methanol or 95% ethanol. When diluted in methanol or ethanol, the solution will not freeze.

[0044] When the 1X X-gal/NMP/methanol is stored for 16 weeks at 4°C, there is no loss in X-gal activity as measured by applying 100 µl to a LB plate, streaking *E. coli* pUC19 transfectant, incubation at 37°C and examination for blue colonies after 16 hours.

[0045] Tables 2 and 3 are showing the results obtained by testing the activity of X-gal in solution with different solvent on a weekly basis. In Table 2, the tested solutions were stored at 4°C. At weekly intervals, 100 µl was applied to an LB+Amp plate and streaked with *E. coli* containing pUC19. The plate was incubated overnight at 37°C. The following morning the plate was examined for blue colonies. In Table 3, LB + Amp plates were prepared and stored at 4°C. At weekly intervals, a plate was removed and streaked with *E. coli* containing pUC19. The plate was incubated at 37°C overnight. The blueness of the colonies was then scored.

Table 2

Weekly Testing of X-gal/NMP/Methanol solution

Week	Test Results
0	+
1	+
2	+
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	+
11	+

Week	Test Results
12	+
13	+
14	+
15	+
16	+
17	+
18	+
19	+
20	+

Table 3

Weekly testing of X-gal/NMP or X-gal/NMP/oil

Date	NMP plate	Oil+NMP plate
0	+	+
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+

Results

Essential Oils

[0046] It was shown that when X-gal was first dissolved in NMP, mixed one to one with essential oil, and then diluted in methanol to 10 mg/ml the mixture would ultimately yield colonies that are darker blue than when X-gal is made up in dimethylformamide.

[0047] This work was repeated and expanded to include other essential oils. The oils tested were: Natural Orange Terpene Solvent (Eco-House);

Blue Gum Eucalyptus Organic (Divine Essense); Atlas cedarwood (Pranarom); Sea Pine Turpentine (Pranarom) and Natural Turpenoid.

[0048] In this experiment, a 100 mg/ml solution of X-gal in NMP was prepared. A one to one mix with each essential oil was then made using this solution. The net X-gal concentration is now 50 mg/ml. This solution was then dilute 5X with 100% methanol such that the final concentration of X-gal is 10 mg/ml. One hundred microliters of this solution is then applied per LB plate.

[0049] As a control, 50 µl of a 20 mg/ml solution of X-gal dissolved in dimethylformamide was applied to one LB plate.

[0050] *E. coli* containing pUC19 was streaked onto each "X-gal spread" LB plate for isolated colonies. The plates were incubated at 37°C overnight. After incubation, the growth on each X-gal plate was scored for blueness. The ranking was as follows (least blue to most blue): A (least); B and X-gal/DMF; C, D and E (most blue).

[0051] The results showed that when either Atlas Cedarwood, Sea Pine Turpenine or Natural Turpenoid were mixed with X-gal/NMP/methanol there was an enhanced blueness of the *E. Coli* pUC containing bacteria.

[0052] During assays with Sea Pine Turpentine, the amount of Sea Pine Turpentine added to the X-gal/NMP solution was serially diluted one in two four times, mixed with methanol and then spread onto LB plates. The amount of X-gal added to each plate was the same. The plates were then streaked with *E coli* containing pUC19 and incubated overnight.

[0053] An examination of the plates showed that the bluest colonies were those obtained with the original amount of Sea Pine Turpentine gave the deepest blue color.

[0054] In the previous experiments, the final X-gal concentration was 10 mg/ml. The concentration of X-gal was reduced to 7.5, 5 and 2.5 mg/ml. Plates were prepared and streaked. The net result was that there was a significant drop in blue coloration when the X-gal was dropped from 7.5 to 2.5 mg/ml. In a preferred embodiment of the present invention, X-gal is in a concentration of 10 mg/ml.

[0055] Ligation assays were performed using lambda DNA digested with PstI and pUC19 digested with PstI and CIAP treated. The completed ligation was transformed into DH5α and plated onto LB plates containing:

(a) X-gal dissolved in Dimethylformamide

(b) X-gal dissolved in NMP and methanol

(c) X-gal dissolved in NMP, Sea Pine Turpentine and methanol

[0056] The plates were incubated overnight. The results for a, b and c are shown in Figs. 2, 3 and 4, respectively. For all three plating, there were both white and blue colonies. Moreover, it is shown that the blues colonies of Fig. 4 are of a more intense blue than the ones of Figs. 2 and 3.

[0057] It is possible to incorporate X-gal directly in molten LB agar. To test this with the X-gal solution of the present invention, 500 ml of LB agar was made, autoclaved and cooled to about 50°C. To this 2 ml of a 10 mg/ml X-gal/NMP/SeaPine/methanol solution was added and mixed. Upon the addition of the X-gal solution, there was a cloudy appearance throughout the agar as it mixed. Mixing for 3 to 4 minutes did not disperse the cloudiness. However, when the plates were poured, the cloudiness dispersed upon cooling and solidification. A slight surface cloudiness was noted several hours later when the plates were inverted for incubation overnight at room temperature. However, the next day the plates looked normal.

[0058] One plate was used to streak a white and a blue colony and incubated overnight at 37°C.

[0059] Two plates were left at 25°C. These two plates served as shipping simulators. The plates were tested after 5 days and were streaked with a blue and white colony and incubated overnight.

[0060] Aging studies have been set up for the liquid product both with and without Sea Pine Turpentine. The studies conducted with the X-gal/NMP/methanol solution shown that the product is stable for 17 weeks at 4°C with only a minor pale yellow color developing over time. This has being conducted at the same time with the X-gal/NMP/SeaPine/methanol product.

[0061] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A non-toxic dipolar solvent for chromogenic substrate for detecting presence of bacteria, which comprises a stabilizing amount of a solubilizing agent.

2. The solvent of claim 1, wherein said solvent is a microemulsion.

3. The solvent of claim 1, wherein said solubilizing agent is at least one selected from the group consisting of 1-Methylpyrrolidone (NMP), N'-dimethyl propylene urea (DMPU), Propylene carbonate (PC) and essential oil.

4. The solvent of claim 1, wherein said essential oil is present in an effective solubilizing concentration for dissolving said chromogenic substrate.

5. The solvent of any one of claims 3 and 4, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

7. A method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce said lac operon.

8. The method of claim 7, said lac operon being induced in one selected from the group consisting of *E. Coli*, Bacillus subtilis, phage, or *in situ* tissues.

9. The method of claim 7, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

10. A method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of said bacteria.

11. The method of claim 10, wherein said essential oil is selected from the group consisting of Abies alba, Aniba roseodora, Cedrus atlantica, Citrus aurantifolia, Citrus aurantium, Citrus bergamia, Citrus limon, Citrus paradisi, Citrus reticulata, Citrus sinensis, Cupressus sempervirens, Juniperus communis, Juniperus virginiana, Picea mariana, Pinus sylvestris, Ravensara aromatica, Rosmarinus officinalis, citrus extracts, pine terpenoids, conifers extracts, limonene oil and linseed oil.

ABSTRACT OF THE INVENTION

The present invention relates to a non-toxic dipolar solvent for chromogenic substrate for detecting presence of bacteria, which comprises a stabilizing amount of a solubilizing agent. The present invention also relates to a method for inducing lac operon in screening assay, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce the lac operon. The present invention further relates to a method for detecting the presence of bacteria, comprising the step of contacting an agar plate with at least one essential oil in a concentration sufficient to induce detection of the bacteria.

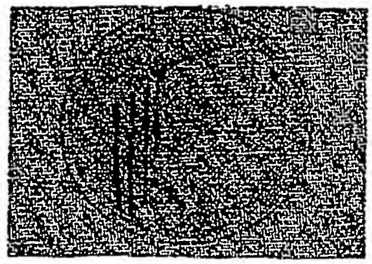


Fig. 1

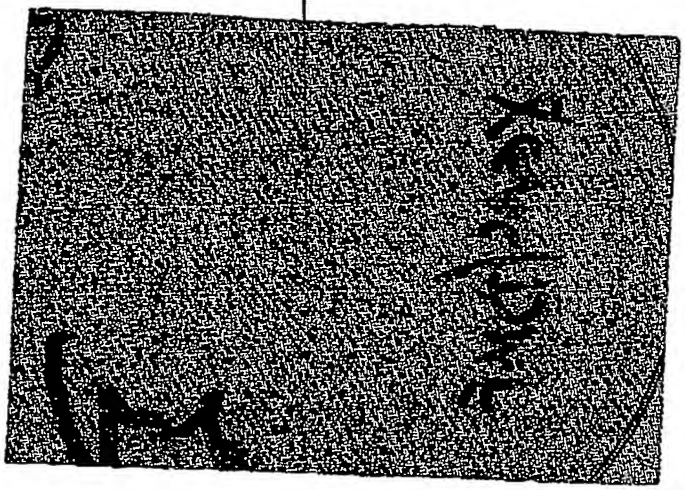


Fig. 2

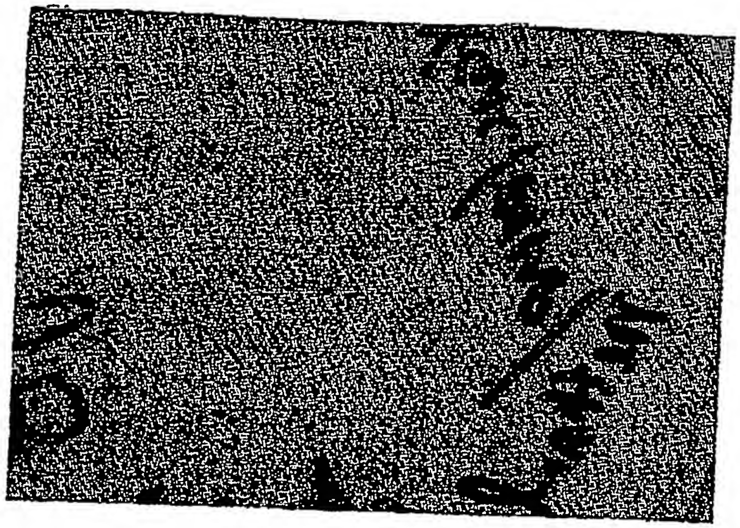


Fig. 3

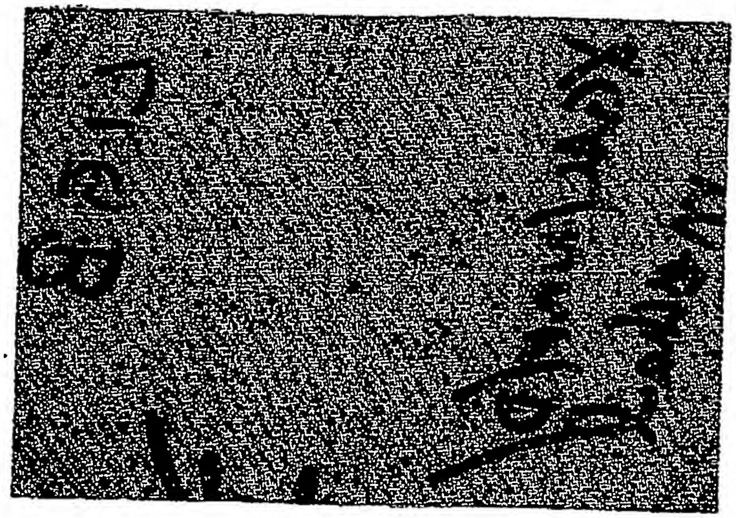


Fig. 4